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Absence of neocytolysis in humans returning from a 3-week high-altitude sojourn

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REGULAR PAPER

ACTA PHYSIOLOGICA

Absence of neocytolysis in humans returning from a 3-week high-altitude sojourn

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Abstract

Aims: Total haemoglobin mass (tot-Hb) increases during high-altitude acclimatization. Normalization of tot-Hb upon descent is thought to occur via neocytolysis, the selective destruction of newly formed erythrocytes. Because convincing experimental proof of neocytolysis is lacking, we performed a prospective study on erythrocyte survival after a stay at the Jungfraujoch Research Station (JFJRS; 3450 m).

Methods: Newly formed erythrocytes of 12 male subjects (mean age 23.3 years) were age cohort labelled in normoxia (110 m) and during a 19-day high-altitude sojourn by ingestion of $^{13}C2$- and ^{15}N-labelled glycine respectively. Elimination dynamics for erythrocytes produced in normoxia and at high altitude were measured by

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isotope ratio mass spectrometry of haem, by determining tot-Hb, reticulocyte counts, erythrocyte membrane protein 4.1a/4.1b ratio and by mathematical modelling.

Results: Tot-Hb increased by 4.7% ± 2.7% at high altitude and returned to pre-altitude values within 11 days after descent. Elimination of ^{13}C- (normoxia) and ^{15}N- (high altitude) labelled erythrocytes was not different. Erythropoietin levels and counts of CD71-positive reticulocytes decreased rapidly after descent. The band 4.1a/4.1b ratio decreased at altitude and remained low for 3-4 days after descent and normalized slowly. There was no indication of haemolysis.

Conclusion: We confirm a rapid normalization of tot-Hb upon descent. Based on the lack of accelerated removal of age cohorts of erythrocytes labelled at high altitude, on patterns of changes in reticulocyte counts and of the band 4.1a/4.1b ratio and on modelling, this decrease did not occur via neocytolysis, but by a reduced rate of erythropoiesis along with normal clearance of senescent erythrocytes.

KEYWORDS

erythropoiesis, high altitude, membrane protein 4.1R, neocytolysis, post-altitude, total haemoglobin mass

1 | INTRODUCTION

An increased haemoglobin (Hb) concentration and total Hb mass (tot-Hb) are typical observations in long-term sojourners at high altitude and in high-altitude residents.[1-4] It is caused by a HIF-2alpha-driven and erythropoietin (Epo)-dependent stimulation of erythropoiesis [5,6] and a decrease in plasma volume,[1] and boosts the oxygen-carrying capacity of blood when the partial pressure of oxygen in inspired air and in blood is low. Upon descent from high altitude, EPO levels fall, plasma volume increases and, subsequently, Hb concentration and tot-Hb normalize within a few days.

Because of the short time required for the readjustment of tot-Hb, it has been claimed that mechanisms other than a decrease in the erythropoietic activity must account for the decrease in tot-Hb. The low number of reticulocytes was taken as evidence for removal, especially, of the young fraction of erythrocytes (neocytes), which resulted in formulating the hypothesis of neocytolysis.[7] The phenomenon was first described in astronauts, where centralization of blood flow seems to cause a decrease in Epo levels.[8] Other supporting evidence was a low reticulocyte count and an altered pattern of the decay rate of ^{51}Cr-labelled erythrocytes.[8] The hypothesis claims that, particularly in the early phase, the decreased Epo triggers the specific removal of young erythrocytes[8] by low Epo-dependent interaction of vascular endothelium and macrophages[9] because high Epo levels would serve as a survival factor not only for erythroid precursors but also for circulating young erythrocytes.[7] A decreased erythropoietic rate was thought to account for low tot-Hb and Hb concentrations during continued low Epo levels. The hypothesis of neocytolysis was subsequently proposed to explain the anaemia of chronic renal failure, where Epo levels are low,[10,11] anaemia of malaria[12] and certain congenital haemolytic anaemia such as pyruvate kinase deficiency.[13] It was also proposed that neocytolysis explains the rapid decrease in tot-Hb upon descent from high altitude, when elevated Epo levels return to normal.[7,14-17] Rice and colleagues[14] found that administering Epo to three subjects upon descent prevented the loss of reticulocytes, indicating that the removal of erythrocytes depended on Epo but not on oxygen availability.

In all these reports, the main evidence supporting neocytolysis is a decrease in the reticulocyte count,[18,19] but solid experimental evidence demonstrating the selective loss of neocytes and an evaluation of the role of decreased erythropoiesis are missing.[18,19] Nevertheless, the neocytolysis hypothesis is acknowledged in editorials[20] and in haematology textbooks.[21]

The most stringent proof of neocytolysis would require demonstration of the clearance of neocytes after a rapid decrease in Epo levels subsequent to descent from high altitude after a prolonged sojourn, for example, by following the fate of an age cohort of young erythrocytes. This technique had been applied but revealed no conclusive results, likely because of insufficient labelling intensity.[14] It was therefore the goal of this study to monitor the survival of age cohorts of erythrocytes metabolically labelled with ^{15}N-glycine during maturation in the bone marrow at high altitude as well as other markers of erythrocyte age upon descent from a 19-days stay at an altitude of 3450 m (Jungfraujoch Research Station [JFJRS]) in comparison to erythrocytes labelled with ^{13}C2-glycine in the pre-altitude normoxia period.

2 | RESULTS

Age cohort-labelled erythrocytes, total haemoglobin mass (tot-Hb), reticulocyte counts and reticulocyte maturity, markers of erythrocyte age, iron metabolism and markers of haemolysis were analysed in 12 healthy male volunteers (age: 23.3 ± 3.3 years; height: 185 ± 7 cm; weight: 75.8 ± 5.9 kg; mean ± SD). Measurements were performed before (in normoxia; white), during a 19-days long sojourn at high altitude (3,450 m a.s.l. at the Jungfraujoch Research Station, Switzerland; blue) and after return to sea level (grey; timeline in Figure 1). Age cohort labelling with glycine, which is incorporated metabolically into haem during synthesis, was performed by feeding the subjects a bolus of ^{13}C2-glycine in the pre-altitude test, and glycine labelled with ^{15}N at high altitude 9 days before return to sea level. Rapid disappearance of the ^{15}N-label from the circulation soon after return of the subjects to sea level would directly indicate that erythrocytes produced and labelled at high altitude are prematurely cleared.

Ascent to high altitude shows the expected increase in Epo plasma levels above baseline (+53%; $P < .001$), which remained elevated at high altitude, although 12% lower on day 18 than on day 10 (Figure 2A). Epo levels dropped at descent from altitude (Figure 2A), and for 1 week remained 50% lower than at high altitude ($P < .001$) and ~30% lower with respect to pre-altitude levels ($P < .001$). The high altitude-induced increase in tot-Hb by 4.7% ± 2.7% (mean ± SD; n = 12) was reversed 11 days after descent (Figure 2B). Plasma volume was decreased by 18.0% ± 8.1% ($P < .001$) on day 18 at the JFJRS compared to pre-altitude, and was still below pre-altitude values 11 days after descent (−8.1% ± 7.9%; $P = .002$), but was significantly increased relative to high altitude ($P < .001$).

The increase in the number of short-lived, early reticulocytes,[22] evaluated as CD71$^+$ and CD235a$^+$, on days 5 (+23%; $P = .037$) and 10 (+29%; $P = .014$) at high altitude (Figure 3A) indicates the stimulation of erythropoiesis. Upon descent, their counts decreased (day 3: −48% vs high altitude, $P = .001$; day 5: −54% vs high altitude, $P = .001$) to values even below baseline (day 3 after descent: −40% vs pre-altitude; $P < .003$), reflecting suppression of erythropoiesis (Figure 3A). The counts of the longer-lived RNA$^+$ reticulocytes were increased at high altitude (day 18: +30%; $P = .001$) compared with baseline (Figure 3B). Their number was still higher than at baseline on day 3 after the descent (+18%; $P = .009$) but then fell below baseline (day 10 after the descent: −26%; $P = .003$). The change in RNA fluorescence intensity (Figure 3C) reflects this differential behaviour of CD71$^+$/CD235a$^+$ and RNA$^+$ erythrocytes. It was highest, when the release of reticulocytes from the bone marrow was highest at the beginning of the stay at high altitude (+53% on day 3 $P < .001$), decreased on day 18 at high altitude (−35%; $P < .001$) and even further decreased 3 days after descent (−41%; $P < .001$).

The highest level of age cohort label appeared in blood 20 to 30 days after glycine intake. However, in contrast to the hypothesis, after return to sea level, the survival of the age cohort of cells labelled at high altitude (Figure 4A, circles) followed exactly the curve obtained in the normoxic pre-altitude test (Figure 4A, triangles). This result clearly demonstrates that the neocytes produced during the stay at high altitude have not been removed but were still present in circulating blood. This interpretation is supported by a mathematical model elaborated to predict the evolution of the age cohort label and of other haematological parameters in the presence or absence of neocytolysis after return to sea level (see Methods and Supplement for details): The

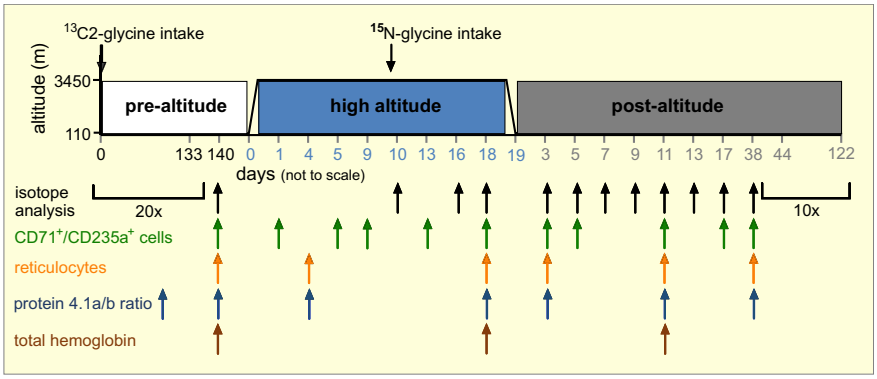

FIGURE 1 Timeline of blood sample collection. Sampling time is colour coded: Sampling during the pre-altitude control study is shown in white, high altitude in blue and collection after return from high altitude in grey. This colour coding is also used for the bar charts. Black arrows indicate blood collection for the measurement of ^{13}C/^{12}C (pre-altitude, 110 m) and ^{15}N/^{14}N (high altitude, 3540 m, and post-altitude; 110 m) in haem. The exact days blood samples were collected after glycine intake for isotope incorporation in the pre-altitude and altitude/post-altitude tests are shown in Figure 4A. The volumes of blood collected at each of the indicated time points were: age cohort labelling (black arrows, 2 mL); CO-Hb/tot-Hb (brown arrows; 2 × 1.5 mL); reticulocyte and erythrocyte functions (not shown here; orange arrows; 30 mL), protein 4.1R (dark blue arrows; 1 mL) and CD71/CD253 (green arrows; 50 µL)

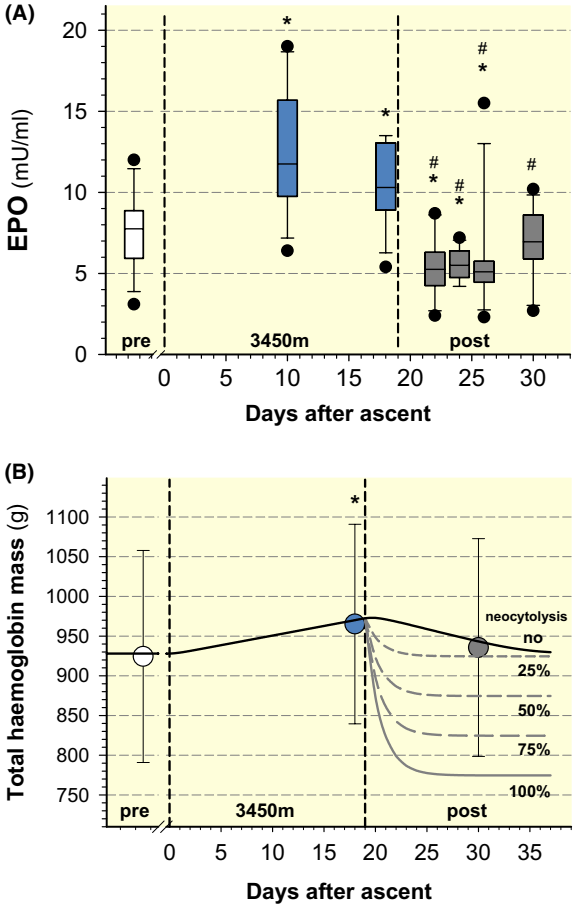

FIGURE 2 Changes in Epo levels and tot-Hb during acclimatization at high altitude and after return to sea level. A, Box plots of plasma levels of Epo measured during the study. B, Mean values ± SD of total haemoglobin mass during and after the sojourn at high altitude measured by CO rebreathing before ascent (pre), on day 18 at high altitude (3450 m) and 11 days after descent (post). The black line indicates the values of tot-Hb predicted by the mathematical model in the absence of neocytolysis. The grey lines indicate that the tot-Hb values predicted in case neocytolysis were triggered at four possible degrees of intensity, upon return to sea level. A and B, Vertical dashed lines indicate the beginning and end of the stay at high altitude. *Significantly different from pre-altitude control; #significantly decreased ($P < .05$) from altitude values

model fits the observed survival curve of ^{15}N-labelled erythrocytes very well when no neocytolysis is assumed (black line in Figure 4A), whereas it predicts a rapid decline in ^{15}N-labelling intensity upon descent if neocytolysis of different degrees had occurred (grey lines in Figure 4A).

Additional evidence for the presence of neocytes in the circulation rather than neocytolysis came from the analysis of protein 4.1R. The 4.1a/4.1b ratio can be considered a 'molecular clock'[23,24] because non-enzymatic deamidation of asparaginyl residues in protein 4.1b, the isoform present in erythroblasts, results in 4.1a in a time-dependent manner, and, thus, the protein 4.1a/4.1b ratio increases with erythrocyte age.[23,24] The mean 4.1a/4.1b ratio of circulating erythrocytes progressively decreased at high altitude (Figure 4B), reflecting the increasing abundance of young erythrocytes in blood with respect to baseline. After descent, the 4.1a/4.1b ratio remained low proving the presence in the circulation of the neocytes produced at high altitude. The model predicts this pattern quite well (black line, Figure 4B) and indicates that neocytolysis would result in a rapid increase in the 4.1a/4.1b ratio to values higher than pre-altitude within a few days after descent because of the removal of erythrocytes high in protein 4.1b (grey line in Figure 4B).

An increased iron demand associated with the stimulation of erythropoiesis is indicated by the decreased plasma ferritin levels on days 10 (−6%) and 18 (−14%) at high altitude ($P < .001$) respectively (Table 1). Ferritin was still low on day 3 after descent (−28% with respect to baseline; $P = .002$) but then returned to the pre-altitude value. Plasma iron levels remained unchanged throughout the study ($P = .079$). There was an increase in transferrin levels at high altitude (+5%; $P = .047$; HA-18), whereas transferrin saturation changed little ($P = .046$). The levels of transferrin decreased below the pre-altitude values upon descent (−5%; $P = .022$, post-altitude day 7). There was no change in total, direct or indirect bilirubin. Haptoglobin increased at high altitude (day 10: +26%, $P = .011$; day 18: +24%, $P = .043$) (Table 1), and slowly returned to normoxic values upon return to sea level.

3 | DISCUSSION

A rapid normalization of tot-Hb has been reported after descent from high altitude.[7,14-16] This could be achieved by a reduction of the rate of erythropoiesis or by an increase in the rate of removing of circulating erythrocytes. The latter has been proposed to occur by the selective elimination of newly formed erythrocytes, that is, 'young' erythrocytes called neocytes.[7] Our results confirm the decreases in tot-Hb and reticulocyte counts within a few days after descent from high altitude, but we did not find indications of haemolysis. In addition, we demonstrate for the first time that the survival of age cohort-labelled erythrocytes produced during a stay at high altitude did not differ from that of erythrocytes produced at sea level, and that only the number of the very young, CD-71-positive fraction of reticulocytes was decreased but that the older reticulocytes remained in circulation. Furthermore, results from modelling strongly support the notion that neocytolysis, the selective removal of young erythrocytes produced at high altitude, cannot explain the observed changes in age cohort-labelled erythrocytes or in markers of erythrocyte age after the descent to sea level, but that decreasing the rate of erythropoiesis is sufficient to explain the decrease in tot-Hb after descent from high altitude.

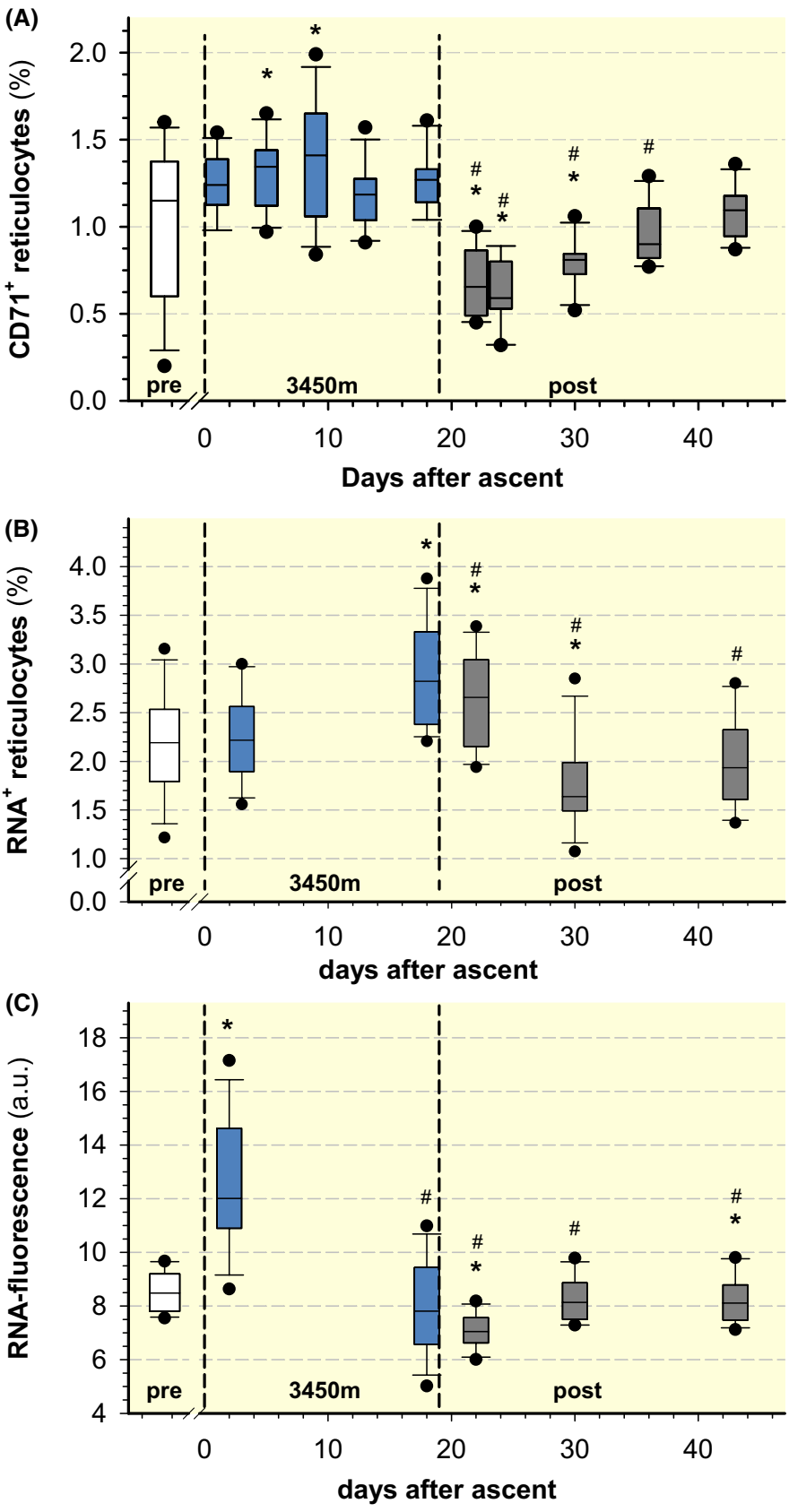

FIGURE 3 Changes in reticulocyte counts and reticulum-fluorescent intensity during acclimatization at high altitude and after return to sea level. Box plots of (A) reticulocyte counts determined as CD71- and CD235a-positive cells, (B) reticulocyte counts determined as RNA-positive erythrocytes and (C) RNA fluorescence intensity in reticulocytes measured by flow cytometry. n = 12. A–C, Vertical dashed lines indicate the beginning and end of the stay at high altitude. *Different from pre-altitude control; #altitude day 19 and post-altitude values different from values on day 4 at high altitude

Age cohort labelling is a strong tool to evaluate survival of erythrocytes produced in special situations. Preferential removal of the young erythrocytes, which were produced during the stay at high altitude, would result in a rapid decrease in the number of age cohort-labelled erythrocytes within a few days after descent. This can be predicted by modelling

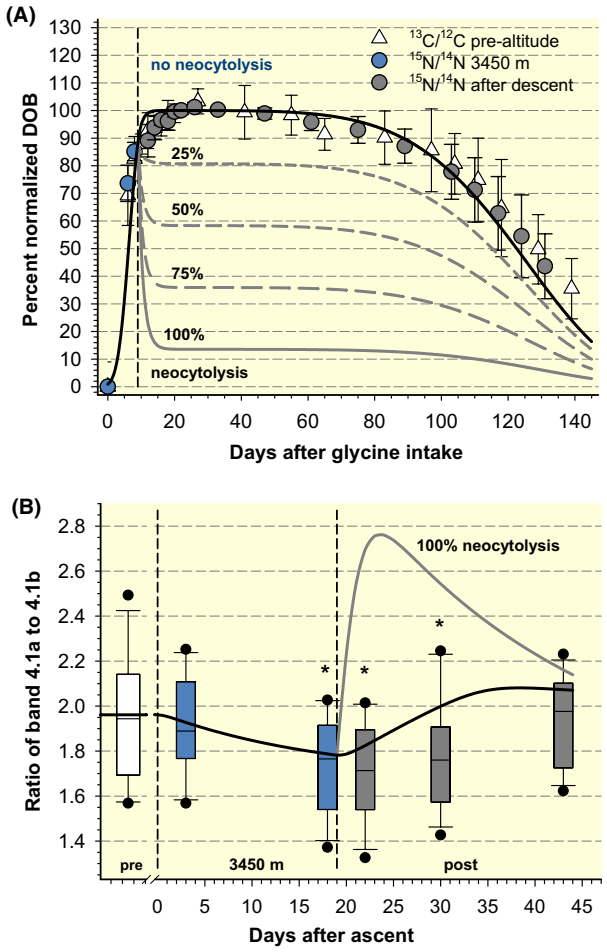

FIGURE 4 Change in age cohort labelling intensity and erythrocyte membrane protein 4.1a/4.1b values during acclimatization at high altitude and after return to sea level. A, Time course of $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios in haem. For the pre-altitude control study, $^{13}C2$-labelled glycine was ingested ~6 months prior to the ascent to high altitude (intake and all time points in normoxia; white triangles). On day 10 at high altitude, ^{15}N-labelled glycine was ingested to label the erythrocytes produced during the stay at high altitude (blue circles). Descent was on day 9 after ^{15}N-glycine intake (grey circles). Individual values for the $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios are normalized to the mean values of delta over baseline (DOB) on days 19-28 after intake. Mean values ± SD, n = 12. Individual curves showing the changes in the $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios are in the Figure S4. The vertical dashed line indicates the end of the stay at high altitude (relevant for $^{15}N/^{14}N$ data). The black line shows the change in age cohort labelling predicted by the model for a normal erythrocyte lifespan (see Methods). Grey lines indicate the predicted change in the $^{15}N/^{14}N$ upon descent if neocytolysis of varying intensity occurred. B, Box plots of the 4.1a/4.1b ratio of band 4.1R measured in erythrocyte ghost membranes before, during and after the stay at high altitude. n = 12. *Different from pre-altitude control, #different from peak value at high altitude. The black line indicates the behaviour in the 4.1a/4.1b ratio predicted by the model if neocytolysis did not occur. The grey line indicates the predicted change if neocytolysis after descent was 100% responsible for the change. Vertical dashed lines indicate the beginning and the end of the stay at high altitude

complete or partial neocytolysis indicated by the grey lines in Figure 4A. Since these lines clearly do not fit the data, the results in Figure 4A and the individual curves shown in Figure S3 indicate that the neocytes produced during the stay at high altitude remained in circulation and that their lifespan was not truncated in comparison to erythrocytes produced in normoxia. Thus, this result does not support the occurrence of neocytolysis. Our results contradict a report on a (statistically not significant) change in age cohort-labelled erythrocytes claiming compatibility with neocytolysis.[14] However, the authors indicate that the labelling intensity may have been insufficient.[14]

Several other parameters evaluated in our study indicated the presence of neocytes in circulating blood after descent, again pointing to the absence of neocytolysis. The level of reticulocytes counted as RNA-positive erythrocytes was still elevated on day 3 after descent (Figure 3B). Because the elimination of RNA from reticulocytes requires several days, this finding indicates that these reticulocytes had not been removed rapidly after descent. The 4.1a/4.1b ratio of the erythrocyte membrane protein 4.1R strongly depends on erythrocyte age.[23] It decreased at high altitude because of the appearance of reticulocytes and young erythrocytes subsequent to a stimulation of erythropoiesis and remained low until day 10 after descent (Figure 4B). These decreased 4.1a/4.1b ratio indicates the persisting presence of the young erythrocytes produced at high altitude in the circulation. If these cells had undergone neocytolysis, one would expect a sharp increase in the 4.1a/4.1b ratio after descent, as indicated by the model (Figure 4B). Together, the results regarding RNA-positive erythrocytes and the band 4.1a/4.1b ratio strongly support the notion that the erythrocytes produced at high altitude remained in the circulation and did not undergo neocytolysis.

The removal of any age cohort of erythrocytes after descent might also cause alterations in parameters indicating haemolysis. Increased plasma levels of haem-breakdown products and elevated levels of ferritin indicating recirculation of released iron have been reported after a return from altitudes >4000 m.[14] Decreased haptoglobin levels might be an indicator of haemolysis. However, we did not observe changes in any of these parameters upon descent that were consistent with haemolysis.[25]

There is of course the possibility that removal of only ~5% of the tot-Hb resulted in minute changes that could not be detected with those parameters. If there were mild haemolysis, our results would not allow the drawing of conclusions regarding the possible mechanisms. A different experimental setting would be needed to test for the altered expression of catalase and increased mitochondrial ROS production by reticulocytes, as found in mice,[26] and for an Epo-dependent interaction of erythrocyte surface adhesion molecules

TABLE 1 Change in laboratory parameters during the stay at the Jungfraujoch Research Station (3450 m) and after the return to Heidelberg (110 m)

	Control	HA-10	HA-18	Post-3	Post-5	Post-7	Post-11	*P*
Serum iron (μmol/L)	22.0 ± 7.1	18.4 ± 7.6	23.6 ± 8.6	28.0 ± 12.7	26.6 ± 14.7	21.2 ± 6.7	18.0 ± 6.2	.079
Ferritin (μg/L)	66.6 ± 53.9	62.6 ± 68.2[a,*]	53.8 ± 47.9[a,*]	47.8 ± 39.8[a,*]	58.1 ± 39.1	68.1 ± 47.5	63.0 ± 39.9	.001[*]
Transferrin (g/L)	2.31 ± 0.18	2.37 ± 0.19[a]	2.42 ± 0.21[a,*]	2.32 ± 0.21[b]	2.22 ± 0.15[b]	2.19 ± 0.08[b,*]	2.30 ± 0.18	.001[*]
Transferrin saturation (%)	38.8 ± 13.9	30.8 ± 12.7[b]	39.3 ± 13.9	48.3 ± 21.1[c]	40.7 ± 13.2	38.5 ± 11.9	31.5 ± 12.5	.046[*]
Haptoglobin (g/L)	0.62 ± 0.32	0.78 ± 0.28[*]	0.77 ± 0.38[*]	0.72 ± 0.30	0.68 ± 0.30	0.71 ± 0.34	0.73 ± 0.34	.036[*]
Total bilirubin (mg/dL)	0.93 ± 0.22	1.01 ± 0.38	1.13 ± 0.52	1.04 ± 0.33	1.02 ± 0.38	1.02 ± 0.35	0.83 ± 0.30	.306
Direct bilirubin (mg/dL)	0.33 ± 0.09	0.37 ± 0.14	0.38 ± 0.16	0.37 ± 0.12	0.37 ± 0.18	0.36 ± 0.12	0.30 ± 0.11	.620
Indirect bilirubin (mg/dL)	0.59 ± 0.16	0.64 ± 0.25	0.75 ± 0.36	0.68 ± 0.23	0.65 ± 0.23	0.66 ± 0.24	0.48 ± 0.25	.074
CRP (mg/L)	2.01 ± 0.03	3.08 ± 3.73	2.24 ± 0.58	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	.459
IL-6 (pg/mL)	3.12 ± 1.70	3.85 ± 1.24	3.67 ± 1.55	3.56 ± 1.39	3.59 ± 1.66	3.47 ± 1.69	3.23 ± 1.02	.901

Note: Mean values ± SD from 12 subjects. HA, high altitude; post-X, X days after descent; CRP, C-reactive protein; IL-6, interleukin-6. Values for CRP < 2 mg/L in the laboratory report were set at 2 for statistical analysis. Level of statistical significance: $P < .05$.

[a]Significant difference compared to post-5.

[b]Significant difference between HA-18 and post-3, 5, 7 for transferrin.

[c]Significant difference between HA-18 and post-3 for transferrin saturation.

*Indicates statistical significance in comparison to pre-altitude control.

with the endothelium and their subsequent removal by macrophages.[7,26,27]

Because our results do not support the facilitated removal of neocytes from the circulation upon descent, that is, neocytolysis, a decreased erythropoiesis rate appears to be the only, and perhaps the most intuitive, mechanism that might explain the decrease in tot-Hb after descent. While we have no direct measure thereof, the post-descent decreases in the number of CD71-positive erythrocytes (Figure 3A) and the rapid decline in reticulocytes with an elevated RNA content (Figure 3B,C) are consistent with this notion. High numbers of both parameters would reflect the presence of very immature reticulocytes because of the stimulation of erythropoiesis.[22] The assumption of a decreased rate of erythropoiesis is supported by previous studies showing a decreased rate of ^{59}Fe removal from the blood in highlanders after descending from high altitude to values well below those in normoxic controls.[28]

Modelling (details in the supplement) based on our data on age cohort labelling and CD71-positive reticulocyte counts revealed that a transient decrease in the erythropoiesis rate by ~35% would be sufficient to explain the normalization of the elevated tot-Hb level within 11 days after descent from high altitude, assuming that the rate of removal of senescent erythrocytes and the erythrocyte lifespan remained constant. The latter assumption is supported by the lack of a difference between the $^{13}C/^{12}C$ and $^{15}N/^{14}N$ data (Figure 4A). Changes in the 4.1a/4.1b ratio estimated with this model showed very good agreement with the observed data (Figure 4B), and are far from those predicted by the model of a neocytolysis scenario (Figure 4B).

4 | CONCLUSIONS

Robust data obtained from age cohort labelling of young erythrocytes produced at high altitude speak against the selective removal of neocytes, and thus against a process of neocytolysis upon return to sea level. Data on changes in reticulocyte counts and of the protein 4.1a/4.1b ratio, which serves as a 'molecular clock' measuring erythrocyte senescence, corroborate this interpretation. Therefore, the observed reduction in tot-Hb after descent from high altitude has to be explained by a reduced erythropoietic rate, at a constant rate of clearance of senescent erythrocytes, which is supported by modelling. Since reducing the erythropoietic rate, and thus in tot-Hb, depends on lowering the Epo levels upon descent, tot-Hb should also decrease rapidly in equivalent situations, such as the cessation of Epo treatment as in renal failure patients, when athletes return from training at high altitude or in patients on long-term oxygen therapy. The time course of the decrease in plasma Epo levels and in the total erythrocyte mass observed during space flight, the condition under which neocytolysis was first proposed,[8] also fits this pattern.

5 | METHODS

5.1 | Study design

Subjects were studied before, during and after a 19-day sojourn at high altitude (3450 m, Jungfraujoch Research Station, Switzerland) to measure changes in total haemoglobin mass (tot-Hb) by CO rebreathing, erythrocyte lifespan by age cohort labelling, erythropoietin levels, iron status and functional parameters characteristic of prematurely senescent erythrocytes that might indicate cell destruction and neocytolysis. Pre- and post-altitude measurements were performed in Heidelberg, Germany (110 m; here referred to as sea level). The study was approved by the ethics committees of the University of Heidelberg, Germany, (S-066/2018) and the University of Bern, Switzerland (2018-01766) and was performed in accordance with the Declaration of Helsinki.

5.2 | Participants

Twelve healthy male subjects (23.3 ± 3.3 years, 185 ± 7 cm, 75.8 ± 5.9 kg) were recruited by public announcement at the University of Heidelberg and participated after providing written informed consent. Subjects were physically fit and had normal haemoglobin (Hb) concentrations and plasma iron status. We chose to study males only to avoid possible effects of the menstrual cycle on Hb and iron metabolism. Furthermore, the Research Station at the Jungfraujoch (JFJRS) accommodates only 12 subjects. Including both sexes would have resulted in subgroups that were too small for statistical comparison.

5.3 | Procedures

5.3.1 | Time course

The time course of the collection of blood samples is shown in Figure 1. The pre-altitude control period started ~6 months prior to exposure to high altitude and lasted for 140 days. After a 1-month break, pre-altitude measurements of tot-Hb were performed. Three days after tot-Hb was measured, the subjects travelled to the JFJRS (3450 m) by train. The descent and return to Heidelberg were also by train. Subjects did not ascend to altitudes above 2000 m during the entire pre- and post-altitude study time. For motivational purposes, during their stay at the JFJRS, the subjects performed moderately intense exercise and hiked to the Mönchsjochhütte (3600 m; one way ~30 minutes; weather permitting) several times per week. In addition, all subjects participated in weekly walking tours that were several hours long accompanied by guides.

During the 19-day period, the subjects were monitored clinically with particular emphasis on the development of acute mountain sickness (AMS; evaluated by the Lake Louise questionnaire and the environmental symptoms questionnaire, ESQ; scores for the quality of sleep were not obtained) and received basic clinical examinations. Possible indications of high-altitude pulmonary oedema (HAPE) were obtained by auscultation and measuring oxygen saturation (SpO_2) with pulse oximetry (LifeSense II, Nonin). One of the subjects showed very mild signs of AMS in the morning of day 2 at the JFJRS as indicated by a Lake Louise score of 3 and an AMS-C score of 0.7. This was likely caused by a mild airway infection indicated by elevated C-reactive protein (CRP) and interleukin-6 (IL-6) levels. His symptoms normalized within the next few days of the stay at the JFJRS without causal treatment. None of the other subjects developed symptoms of AMS or any illness during the stay at high altitude. This was surprising because the literature indicated a prevalence of AMS of ~35% at the JFJRS.[29,30] There was no evidence of HAPE.

5.3.2 | Measurement of the erythrocyte lifespan

Age cohort labelling of erythrocytes was performed by the ingestion of glycine labelled with non-radioactive, stable isotopes. In the pre-altitude test, subjects ingested 2 g of $^{13}C2$-labelled glycine (CLM-136-MPT-0; 99% $^{13}C2$; Eurisotop, Saint-Aubin, France) dissolved in water and diluted with orange juice after a pre-test blood sample (2 mL, K-EDTA for anti-coagulation) was collected from an antecubital vein using the Sarstedt system. Similarly, 9 days before descending from the JFJRS, the subjects ingested 2 g of ^{15}N-labelled glycine (NLM-202-MPT-0; 98% ^{15}N; Eurisotop). The different label was chosen to avoid blurring by isotope recycling. The days on which blood was drawn for the detection of label intensity in circulating erythrocytes are marked in Figures 1 and 4A.

5.3.3 | Preparation of haem and isotope analysis

Haem was prepared from 0.5 mL of frozen erythrocytes according to the method described by Ramsey,[31] and the $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios were determined according to Browne and colleagues using online-combustion and continuous-flow isotope ratio mass spectrometry[32] calibrated against international standards. For each participant, the individual delta over baseline (DOB) values normalized to the mean value of the highest label intensities (days 19 to 28 after glycine

intake) allowed the comparison of the $^{13}C/^{12}C$ and $^{15}N/^{14}N$ curves. Details are provided in the Supplement.

5.3.4 | Total haemoglobin mass

Tot-Hb was measured with a modified version of a carbon monoxide (CO) rebreathing technique [33] using an automated system (OpCO, Detalo Health, Denmark). The percent carboxy-Hb (% CO-Hb) and Hb concentration (Hb) were measured with a blood gas analyzer (RapidPoint 500, Siemens, Germany). The change in the % CO-Hb between the first and second measurements was used for the calculation of tot-Hb after correction for the amount of CO that remained in the rebreathing system.[34]

5.3.5 | Other measurements

The parameters characterizing iron metabolism (plasma iron concentration, ferritin and transferrin), erythropoietin, parameters indicative of erythrocyte destruction (haptoglobin and bilirubin) and inflammation markers (CRP and IL-6) were measured by the Central Laboratory of the University Hospital, Heidelberg, Germany.

Early reticulocyte counts were determined by flow cytometry (CyFlow Cube6, Sysmex) from blood collected by finger prick, and measurements were performed within 1 hour. Analysis was performed by counting the cells positive for staining of the transferrin receptor (CD71-FITC, CyFlow, Sysmex) and glycophorin A (CD235a-APC, BD Biosciences). Total reticulocyte counts from blood collected from an antecubital vein were measured as RNA-positive cells (Retic-Count™, BD) by flow cytometry (Gallios Flow cytometer, Beckman Coulter Life Sciences).

The ratio of the 4.1a to 4.1b forms of the erythrocyte membrane protein band 4.1R was determined as a surrogate of changes in mean erythrocyte age because of its strong dependence on erythrocyte senescence.[23] Contaminating leucocytes were removed by filtration, and intensities of the doublet of bands 4.1a and 4.1b were measured after the separation of erythrocyte ghost membrane proteins by sodium dodecyl sulphate gel electrophoresis and Coomassie blue staining.[35]

5.4 | Statistical analysis and modelling

The results are shown as box plots and/or as the mean values ± standard deviations. The statistical significance of changes in parameters was calculated by one-way analysis of variance for repeated measurements. Pairwise multiple-comparison procedures were performed using Student–Newman–Keuls tests. Calculations were performed with SigmaPlot software (Systat, Germany).

We modelled (details in the Supplement) the temporal evolution of tot-Hb, the reticulocyte counts, the $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios and the 4.1a/4.1b ratio to estimate the possible contribution of neocytolysis. Modelling was performed with MATLAB (R2020a; version 9.8.0.1323502; The Mathworks Inc, Natick, MA, USA). The data on age cohort labelling were used to determine the erythrocyte lifetime distribution based on a Weibull distribution.[36,37] Changes in the production rate of early erythrocytes over time were modelled on the basis of the increase in the counts of early reticulocytes shown in Figure 3A, that is, a 30% increase upon ascent to high altitude, and a rapid but transient decrease in the erythropoiesis rate by ~35% below the pre-altitude rate and a gradual return to normal following the pattern of change in CD-71-positive reticulocytes (Figure 3A).

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CONFLICT OF INTEREST

We declare that we have no competing interests.

AUTHOR CONTRIBUTIONS

AB, GM, HM and LK made substantial contributions to the conception or design of the work. AS, AvC, AB, AM, CL, ChB, ES, GM, GS, HM, HuM, LH, LK, LS, MK, SF, SiF, SR, StR and TH gave final approval for the manuscript to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. AM, AS, ChB, ES, GM, GS, LH, LK, LS, MK, SF, SR, StR and TH acquired, analysed or interpreted data for the article. AB, CL, GM, HM, LK, SR and StR critically

revised the manuscript for important intellectual content. HM drafted the manuscript and prepared the final draft. HM (corresponding author) had full access to all data and had the final responsibility for submission.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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